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Antibody-Free Virion Titer Greatly Differs Between Hepatitis C Virus Genotypes

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Hepatitis C virus (HCV) virions have been shown to be bound to antibodies in patients with chronic HCV infection. The sera from patients infected with genotype 1b HCV contained more antibody-free virions than those from patients with genotype 2a/2b HCV. When compared at the same levels of serum HCV RNA, free virion titers of genotype 2a/2b-infected patients were much lower than those of genotype 1b-infected patients, indicating that a larger fraction of HCV virions are bound to antibodies in the former than in the latter. The gene segments encoding the hypervariable region (HVR) 1, a principal neutralization epitope, of HCV were amplified from the patients' sera by reverse transcription-polymerase chain reaction. The majority of genotype 2a/2b-infected patients had very similar HVR 1 sequences to one another, whereas patients infected with genotype 1b HCV had highly heterogeneous sequences. Differences in the amount of antibody-free virion and HVR1 sequence variability between genotypes may have an implication in HCV pathogenesis. ***J. Med. Virol. 61:37–43, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: interferon alfa; immune complex; HVR; immunoprecipitation

INTRODUCTION

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, commonly causes post-transfusion and sporadic non-A, non-B hepatitis [Choo et al., 1989; Kuo et al., 1989]. Chronic infection often ensues, leading to serious liver diseases including cirrhosis and hepatocellular carcinoma [Kiyosawa et al., 1990]. Because of a lack of the efficient cell culture system, the mechanisms of viral entry and replication of HCV remain to be clarified. Recently, several groups have reported the cultivation of HCV in cultured cells [Shimizu et al., 1993; Lanford et al., 1994; Kato et al., 1995], but detection of viral replication relied on highly sensitive polymerase chain reaction (PCR). HCV seems to replicate in peripheral blood mononuclear cells as well as in the liver of infected individuals [Zignego et al., 1992; Muller et al., 1993; Lerat et al., 1996].

The HCV genomes are characterized by a marked sequence heterogeneity among isolates. The sequence variability has been used to group HCV isolates into several genotypes [Enomoto et al., 1990; Chan et al., 1992; Simmonds et al., 1994]. The variability is not evenly distributed throughout the genome, and a region of 25 to 30 amino acid residues, located at the N-terminus of the E2 envelope protein, exhibits the highest diversity, thus called the hypervariable region (HVR) [Weiner et al., 1991]. Furthermore, individual patients with chronic infection usually possess several quasispecies clones, and dominant clones change over the course of infection [Martell et al., 1992]. The sequence changes are most frequently found in the HVR, suggesting that the HVR contains the principal epitopes for neutralizing antibodies [Zibert et al., 1995]. HCV virions have been shown to form immune complexes with antibodies in the plasma of patients with chronic infection [Thomssen et al., 1993], and those virions bound to antibodies were reported to have a low infectivity in vivo and in vitro [Hijikata et al., 1993; Farci et al., 1994; Shimizu et al., 1994].

Currently, interferon (IFN) is the only drug for chronic HCV infection, that can induce viral clearance and a marked biochemical and histological improvement in some of the patients. Different HCV strains vary in their responsiveness to IFN therapy, and genotype 1b HCV strains, the most common genotype, are more resistant than genotype 2a and 2b strains [Mita et al., 1994]. Recently, the attachment of HCV in patient's sera to a human T cell line has been shown to be

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the most reliable predictive factor for the response to IFN therapy [Kimura et al., 1998].

In this study, the free virion titers and sequences of the HVR were compared between genotype 1b-infected and genotype 2a/2b-infected patients. The results showed that the sera from patients infected with genotype 2a/2b contained less antibody-free virions than those from patients with genotype 1b HCV, possibly accounting for the difference in the response to IFN therapy between the genotypes. The study also showed that genotype 1b HCV had more diversity in the HVR sequences between patients, as compared with genotype 2a/2b HCV.

MATERIALS AND METHODS

Patients

Seventy-one patients with chronic HCV infection were studied who had elevated levels of serum alanine aminotransferase for more than 6 months, and histologically proven chronic hepatitis. They were positive for serum anti-HCV antibodies and serum HCV RNA, and showed no evidence of hepatitis B virus infection, alcoholism, autoimmunity, or any other type of liver disease. At the time of examination, the patients had not received any anti-HCV drugs including interferon. The duration of infection was not known unless the patients acquired HCV through blood transfusion. Patient's sera were collected and immediately stored at −80°C until use.

Serum HCV RNA and HCV Genotype

The serum HCV RNA levels were determined by a competitive reverse transcription-polymerase chain reaction (RT-PCR) assay [Kato et al., 1993]. The HCV genotype was determined by the RT-PCR with type-specific primers according to the method of Okamoto et al. [1992b].

IFN Therapy

IFN therapy was performed as described previously. [Kimura et al., 1998]. The complete responders to IFN therapy were defined as those with the absence of serum HCV RNA by nested RT-PCR both during the therapy and 6 months after the completion of the therapy. The non-responders had detectable HCV RNA in serum by RT-PCR at the end of the treatment. Non-responders included the transient responders who showed an initial reduction of HCV RNA with subsequent relapse within 6 months after the completion of the therapy.

Immunoprecipitation and Titration of Antibody-Free Virions

HCV virions in circulating immune complexes were immunoprecipitated as described by Hijikata et al., with a slight modification [Hijikata et al., 1993]. Ten μl of the serum was mixed with 100 μl of an undiluted immunoglobulin (Ig) G fraction of goat anti-human Igs (IgA+IgG+IgM) (Organon Teknika Co., NC). The mixture was incubated overnight at 4°C, and then separated into supernatant and pellet fractions by centrifugation at 680 × *g* for 15 min. Nucleic acids were extracted from the supernatant using a nucleotide extraction kit (SMITEST, Sumitomo, Japan), and then serially diluted. An aliquot from the supernatant was tested by the nested RT-PCR for the presence of HCV RNA as described previously [Ito et al., 1996]. The primers used were from the 5′ non-coding region of the HCV genome [Nakatsuji et al., 1992]. The 1st sense primer was 5′ ACTCCACCATAGATCACTCC 3′; the 1st anti-sense primer was 5′ AACACTACTCGGCTAGCAGT 3′; the 2nd sense primer was 5′ TTCACGCAGAAAGCGTCTAG 3′; and the 2nd anti-sense primer was 5′ GTTTATCCAAGAAAGGACCC 3′. The amplified DNA was visualized by agarose gel electrophoresis (2%) and ethidium bromide staining. The free virion titer was determined from the maximum dilution of the supernatant nucleic acids in which HCV genome could be detected, and was expressed as values/ml. When the mean of the free virion titers was calculated (Fig. 1), we considered the titer under the limit of detection to be zero.

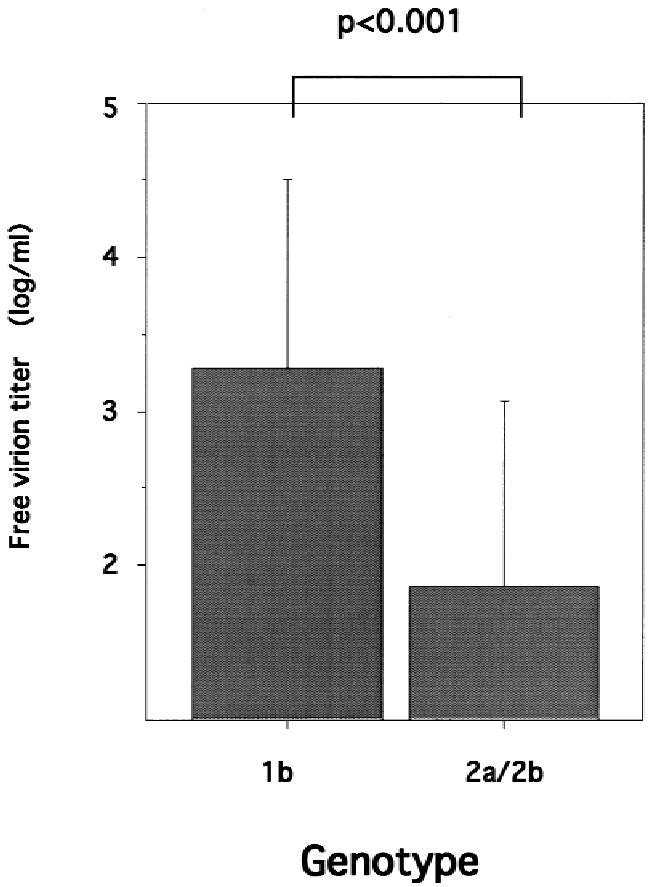

Fig. 1. Comparison of the free virion titer between patients infected with HCV genotype 1b and with genotype 2a/2b. The free virion titers in the sera from patients with chronic HCV infection (1b:36 patients and 2a/2b:35 patients) were determined, and expressed as values/ml. Vertical bar in each column indicates the standard deviation. The Fisher's exact test was performed to assess the significance of differences.

Determination of the HVR 1 Sequence of HCV

The sera from 38 patients randomly selected from the total 71 patients were used for the determination of the HVR 1 sequence. Genotype 1b strains contain two HVRs, HVR 1 and HVR 2, whereas genotype 2a/2b strains have only one HVR (HVR1) [Hijikata et al., 1991; Kato et al., 1992]. Nucleic acid was extracted from 100 μl of the serum using an extraction kit (SMITEST; Sumitomo, Japan), and dissolved in 50 μl of distilled water, 10 μl of which was used for RT-PCR to amplify the E2/NS1 region of HCV [Moribe et al., 1995]. Primers for RT and the 1st-round PCR were S1 (5′-TGGCTTGGGATATGATGATGAAC-3′, sense, nucleotide (nt) positions 1277–1299 of HC-J4 [Okamoto et al., 1990]), A1 (5′-GGGGTGAAGCAaTACACTGGaCCaCA-3′, anti-sense, nt 1831-1856 of HC-J4) and A2 (5′-GGGGTGAAGCAgTACACTGGgCCgCA-3′, anti-sense, nt 1839–1864 of BK [Takamizawa et al., 1991]). Primers for 2nd-round PCR were S2 (5′-TGGGATATGATGATCAACTGGTC-3′, sense, nt 1282–1304 of HC-J4), A3 (5′-GTGAAGGAATTCACTGGaCCaCACAC-3′, anti-sense, nt 1828–1853 of HC-J4) and A4 (5′-GTGAAGGAATTCACTGGgCCgCACAC-3′, anti-sense, nt 1836–1861 of BK) [Moribe et al., 1995]. A1/A2 and A3/A4 were produced by mixing residues in the parts indicated by small letters. The nested PCR products of the HVR1 were used directly for fluorescence-based sequencing reactions using a Big Dye terminator cycle sequencing reaction kit (Applied Biosystems, Foster City, CA) under the conditions recommended by the supplier. Primers for the sequencing reaction were HS (5′-GCCTTGCCTACTATTCCATG-3′, sense, nt 1405–1424 of HC-J4) and HA (5′-TTGATGTGCCAACTGCCATT-3′, anti-sense, nt 1581–1600 of HC-J4) [Lanford et al., 1994]. Sequence was read using ABI PRISM 310 DNA sequencing system (Applied Biosystems, Foster City, CA). Sequence analysis was performed using Gene Works software system (IntelliGenetics, CA).

Statistics

In the univariate analysis, the Fisher's exact test and Student's *t*-test were performed to assess the significance of differences in proportions. The Spearman rank correlation coefficient was used to estimate the magnitude of the correlation between the free virion titer and the serum HCV RNA level. Statistical significance was defined as $P < 0.05$.

RESULTS

Difference in the Amount of Antibody-Free Virion Between Genotypes 1b and 2a/2b.

During a previous study to identify reliable predictive factors for successful IFN therapy [Kimura et al., 1998], it was noted that some parameters significantly differed between patients infected with different genotypes of HCV. We reexamined one of those parameters, the titer of antibody-free virions, in a larger number of patients with chronic HCV infection. The clinical characteristics of the patients examined were shown in Table I. There was no significant difference in sex, the level of alanine aminotransferase, liver histology, and the serum viral load, between patients infected with genotype 1b HCV and those with genotype 2a/2b HCV. The sera were separated, by immunoprecipitation with anti-human Ig, into the supernatant fractions containing free virions and the pellet fractions containing antibody-bound virions. Then, nucleic acids were isolated from the supernatant, serially diluted, and examined for the HCV RNA by RT-PCR. Results showed that patients infected with genotype 1b HCV had significantly higher free virion titers than those with genotype 2a/2b HCV ($P < 0.001$) (Fig.1).

TABLE I. Characteristics of Patients With Chronic HCV Infection

Genotype	1b	2a/2b	*P*-value
n	36	35	
Age (yr)	48.8 ± 11.0	43.1 ± 10.4	0.0277
Gender (F/M)	9/27	4/31	0.2189
ALT (IU/l)[a]	133.9 ± 126.6	125.8 ± 103.6	0.7679
Liver histology[b]			
CPH	5	1	0.3821
CAH2A	25	27	
CAH2B	6	7	
Viral load (log copies/50 μl)	4.72 ± 1.12	4.94 ± 0.95	0.4444

[a]ALT, alanine aminotransferase. Normal range, to 38 IU per liter.
[b]Former European classification. CPH, chronic persistent hepatitis; CAH, chronic active hepatitis.

The free virion titers were plotted against the levels of serum HCV RNA. The free virion titers directly correlated with HCV RNA in patients infected with genotype 1b HCV ($r = 0.802$, $P < 0.001$) (Fig. 2A). In contrast, among patients infected with genotype 2a/2b HCV, only a weak correlation was observed ($r = 0.400$, $P = 0.020$), and there were many cases who had high levels of HCV RNA but low titers of free virions (Fig. 2B). When compared at the same levels of HCV RNA, free virion titers of genotype 2a/2b-infected patients tended to be much lower than those of genotype 1b-infected patients. Thus, a larger fraction of HCV virions appear to be complexed with antibodies in genotype 2a/2b-infected patients than in genotype 1b-infected patients.

IFN responsiveness of each patient was also indicated in Figure 2. The complete responders had lower free virion titers than non-responders in each genotype. When all the patients were combined regardless of the genotype, the free virion titer highly correlated with the response to IFN ($P < 0.001$).

Sequence of the Hypervariable Region of HCV

Because HCV virions complexed with antibodies were reported to have a low infectivity [Hijikata et al., 1993; Farci et al., 1994; Shimizu et al., 1994], a significant portion of anti-HCV antibodies should have the neutralizing activity. The HVR1, located in the E2 glycoprotein of HCV, has been shown to contain a major epitope for neutralizing antibodies [Zibert et al., 1995]. Therefore, an analysis was carried out to determine

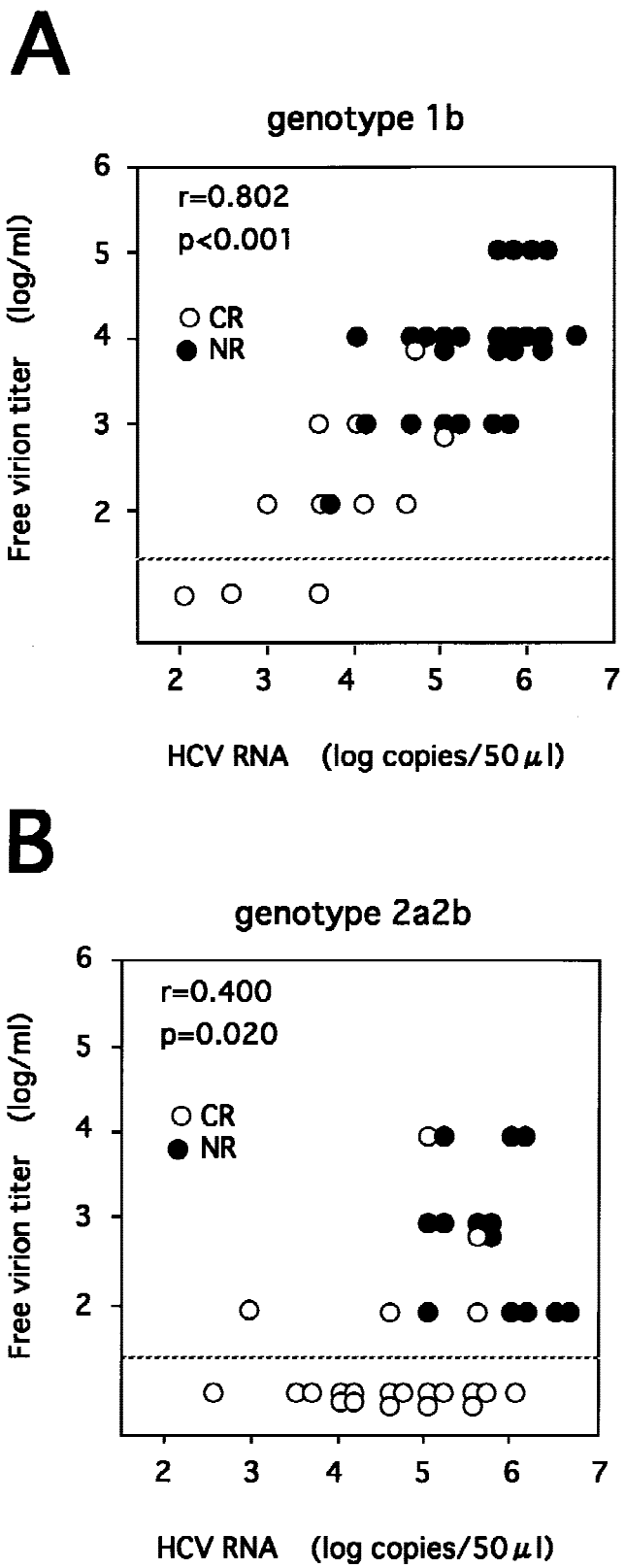

Fig. 2. Correlation between the serum HCV RNA level and the free virion titer in patients infected with genotype 1b **(A)** and with genotype 2a/2b **(B).** The free virion titers were plotted against the levels of serum HCV RNA. The serum HCV RNA was determined by a competitive RT-PCR assay. CR, complete responder; NR, non-responder. The dotted line represents the lower limit of detection of the free virion titration. The Spearman rank correlation coefficient was used to estimate the magnitude of the correlation.

whether there was any characteristic structure within the HVR1 of each genotype that may influence the quantity of free virion.

The sequence encoding the HVR1 of HCV was amplified by RT-PCR from the sera of 38 patients selected at random (1b; 22 patients and 2a/2b; 16 patients). The amplified products were sequenced directly without cloning into plasmid. Figure 3 shows alignments of amino acid residues in the HVR1 deduced from the nucleotide sequences of the PCR products. Patients were arranged in the order of free virion titer. No characteristic amino acid sequences were found in the HVR1 of genotype 2a/2b strains that could differentiate patients with low free virion titers from those with high titers (Fig. 3A). The majority (14/16) of genotype 2a/2b-infected patients, regardless of the free virion titer, had the sequence very similar (≤4 amino acids difference) to the consensus sequence (that was obtained using consensus cut off = 50%). This consensus sequence was also used for the comparison of the sequences of genotype 1b strains. Four out of six genotype 1b-infected patients with the free virion titer of $\leq 10^{2.0}$/ml had the sequence identical or very similar to the consensus (Fig. 3B). In contrast, all but one patients (15/16) with the free virion titer of $>10^{2.0}$/ml had sequences greatly different from the consensus. In addition, the sequences in those patients were highly heterogeneous.

DISCUSSION

In this study, it was shown that the sera from patients infected with genotype 2a/2b HCV had significantly less antibody-free virions than those from patients with genotype 1b HCV, suggesting that genotype 2a/2b virions are more likely to form immune complexes than genotype 1b virions. Furthermore, the majority of patients infected with genotype 2a/2b HCV had the similar sequences in HVR1 to one another, whereas those infected with genotype 1b HCV had highly heterogeneous sequences.

Yoshioka et al. [1997] expressed as glutathione s-transferase fusion protein the HVR sequence from the sera of patients with chronic HCV infection, to assess anti-HVR antibody in the patients' sera. They found that both the incidence of fusion proteins positive for autologous anti-HVR antibody and the activity of antibody were significantly higher in genotype 2a-infected patients than in genotype 1b-infected patients. Our results were consistent with their findings, although antibody response was not necessarily directed against HVR in our subjects.

It has been shown that genotype 1b HCV strains are more resistant to IFN therapy than genotype 2a/2b strains [Hino et al., 1994; Mahaney et al., 1994]. Our finding may provide an explanation for the difference in the response to IFN between the genotypes. Studies on HCV dynamics during therapy have indicated that IFN acts either by inhibiting directly HCV virion production or release [Neumann et al., 1998] or by inhibiting de novo infection of susceptible hepatocytes [Zeuzem et al., 1996, 1998]. The ability of genotype 1b HCV

A

genotype 2a2b

	384 ... 410	free virion titer(log/ml)
consensus	ATYTTGGSAAYTVSRYTSIFASGPSQN	
patient #		
1	S.RI...QV.QNTRGLI.L....SA.K	<2.0
2	.....P..	<2.0
3	.....G.....P.....	<2.0
4	.....A.....	<2.0
5	.....R.....	<2.0
6	T.....NA.....Y.....	<2.0
7	...A.....D.....	<2.0
8	...V.....R..F.....	2.0
9	H.HI...V..QNAA.FV.LLDI....K	2.0
10	.....L.....	3.0
11	.....	3.0
12	.....R.	3.0
13	....A.....E....	3.0
14	.....S.....PP.	3.0
15	...P.....C.....	4.0
16	.....M.....	4.0

B

genotype 1b

	384 ... 410	free virion titer(log/ml)
consensus	ATYTTGGSAAYTVSRYTSIFASGPSQN	
patient #		
17	H.....KPMSPL.LI...T....NH	<2.0
18	.....	<2.0
19	.....G...W....M.....	<2.0
20	R.HV..AQ.GR.AHSL..L.SP....K	2.0
21	.....	2.0
22	.....I...	2.0
23	.....	3.0
24	T.SI..TRTG. AANKIAGF..P.AA.R	3.0
25	Q.....Q..S.TQSFV.LL.P....E	3.0
26	D.HVM..T....TNSL.GL.SQ..A.K	3.0
27	Q.R.L..T..H.TY.LA...SP.A...	3.0
28	E.HV...AT.RSTFQLI.L.K..A..K	3.0
29	T.FV...IV.QATN.F.GF.SA.SA.K	4.0
30	N.RV...TQ.F.TQGLV.F..P..K.K	4.0
31	T..VS. .A.SHAT.GLA.L.SA.S..K	4.0
32	R.HV...T.GR.T.GIMTL..H..Q.K	4.0
33	R.T..I.GK.ASTHSL. .S.HR.AT..	4.0
34	H..V..AVPGRG..SFA.L..P.S...	4.0
35	E.H....Q.GK.AYGLV.L.K.....K	5.0
36	H.H....VV.QNAA.LA.L.NL....T	5.0
37	S.RI...QV.QNTRGL..L.....A.K	5.0
38	T.TV...AT.HNTLGL..L.SF.A..K	6.0

Fig. 3. Amino acid sequences of the HVR1 of the major HCV clones in the sera from patients infected with genotype 2a/2b **(A)** and with genotype 1b **(B).** Gene segments encoding the HVR1 were amplified by RT-PCR, and amino acid sequences of HVR1 (position 384 to position 410) were determined. The consensus sequence was obtained for 2a/2b-infected patients using consensus cut off = 50%, and shown on the top using the single-letter amino acid code. The same consensus sequence was also shown on the top of the sequences of genotype 1b-infected patients. Amino acid sequences were shown with the single-letter amino acid code where residue was different from the consensus sequence. A dash indicates that the residue was identical to the consensus sequence. Patients were arranged in the order of the free virion titer in each genotype.

to better escape complex formation with antibodies (especially neutralizing antibodies), as suggested in our study, may result in higher rates of de novo infection of hepatocytes, which will in turn lead to the production of more virions. Patients infected with genotype 1b HCV may have higher effective (antibody-free) viral loads responsible for de novo infection of hepatocytes, even if they have the similar levels of HCV RNA in sera to those with genotype 2a/2b. Thus, patients with genotype 1b HCV may require more action of IFN to clear the virus, and be more likely to show the resistance to IFN therapy.

What is the explanation for the great heterogeneity of HVR among genotype 1b-infected patients? Infidelity of viral RNA polymerase and immune-driven selection appear to produce the sequence diversity of RNA viruses [Holland et al., 1992]. Many of the diverse genotype 1b HVR sequences may have been selected during the evolutional history of this genotype. These sequences may not induce strong antibody response by nature, thus not forming immune complexes. Alternatively, these sequences may reflect accumulation of escape mutations within individual patients, [Ogata et al., 1991; Okamoto et al., 1992a], and RNA polymerase of genotype 1b HCV may have greater infidelity than that of genotype 2a/2b HCV. It is also possible that the HVR1 consensus sequence observed in the majority of genotype 2a/2b-infected patients as well as some of genotype 1b-infected patients with low free virion titers ($\leq 10^{2.0}$/ml) may have the structural constrain that restricts development of mutations, whereas the sequences found in the majority of genotype 1b-infected patients may be able to mutate without such constrain.

It has been suggested that the early appearance of anti-HVR1 antibodies against N-terminus of HVR1 is associated with acute self-limiting infections of HCV [Zibert et al., 1997]. In contrast, Shimizu et al. localized the epitope for neutralizing antibody to C-terminus of HVR1 [Shimizu et al., 1996]. It should be noted, however, that sequences other than HVR1 may also act as major epitopes for antibody response, affecting immune complex formation, because the identical HVR1 sequence was observed in two patients with low free virion titer (Patient 18 and 21) and another two patients with high titers (Patient 11 and 23). Further, a recent report demonstrated that the ratio of nonsynonymous to synonymous substitution (reflecting the immune pressure) within E1 was higher among those who cleared the viremia, whereas the ratio within HVR1 was higher among individuals with persistent HCV infection, suggesting that HVR1 may act as an immunological decoy, not as an epitope truly involved in HCV neutralization [Ray et al., 1999].

Although further studies are required to clarify the exact mechanism, the difference in the amount of antibody-free virions and sequence variability of HVR should bear on the pathogenesis of HCV infection and contribute to the difference in the response to IFN between the HCV genotypes.

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